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A Motoneuron-Selective Stop Signal in the Synaptic Protein S-Laminin

Brenda E. Porter, Joachim Weis,* and Joshua R. Sanes
Department of Anatomy and Neurobiology
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

Motor axons preferentially reinnervate original synaptic sites on denervated muscle fibers. We have shown that components of synaptic basal lamina direct this selectivity, and we identified a protein, s-laminin, that is concentrated in synaptic basal lamina. Here, we report that a recombinant s-laminin fragment inhibits neurite outgrowth promoted by laminin. A tripeptide sequence in this fragment, Leu-Arg-Glu (LRE), contributes to this inhibition and is itself sufficient to inhibit outgrowth. LRE-mediated inhibition is selective for motoneuron-like cells and is observed in mixtures with several, but not all, outgrowth-promoting substrates. Growth cones extending on laminin stop for up to several hours upon contacting deposits of the s-laminin fragment. Thus, LRE may serve as a cell type–selective and context-dependent target-derived signal that plays a role in synapse formation.

Introduction

When a growing axon encounters its synaptic target, it undergoes a series of local changes that culminate in its transformation into a nerve terminal. Studies in vitro have demonstrated that growth cones acquire new physiological and morphological properties soon after they contact target cells or target-derived membranes, suggesting that components of the target cell surface play important roles in directing this differentiation (Baird et al., 1992a, 1992b; Buchanan et al., 1989; Connor and Smith, 1994; Cooper and Smith, 1992; Dai and Peng, 1993; Dailey and Smith, 1993; Dan and Poo, 1994; Funte and Haydon, 1993; Hall and Sanes, 1993; Tuttle, 1985). However, in contrast to the numerous membrane- and matrix-associated molecules that are believed to direct axons toward a target, few cell surface molecules have been identified that influence the behavior of axons once they have reached their targets.

It seems likely that some of the factors that direct presynaptic differentiation at the neuromuscular junction reside in the basal lamina of the synaptic cleft. Motor axons preferentially reinnervate original synaptic sites on denervated muscle fibers. The structure that axons initially contact at these sites is synaptic basal lamina. Moreover, regenerating motor axons selectively reinnervate at synaptic sites on basal lamina "ghosts" from which muscle fiber cytoplasm and membrane have been removed by damage-induced necrosis and phagocytosis (Sanes et al., 1978; Glicksman and Sanes, 1983). One of the molecules that may direct this selectivity is s-laminin (also called laminin β2; Burgeson et al., 1994), a homolog of the laminin B1 (or β1) chain (Hunter et al., 1989a). S-laminin is concentrated in synaptic portions of the basal lamina sheath of the muscle and bears a site that is selectively adhesive to motoneuron-like cells (Hunter et al., 1989b, 1991). A major determinant of this site is the tripeptide, Leu-Arg-Glu (LRE). Adhesion of motoneuron-like cells to a s-laminin fusion protein is inhibited by LRE in the culture medium, and these cells adhere to short LRE-containing peptides immobilized on the substrate. LRE occurs three times in the rat s-laminin sequence but not at all in laminin B1, for which s-laminin is the synaptic substitute (Sanes et al., 1990). Moreover, several other proteins concentrated in synaptic extracellular matrix also contain LRE in their sequences: agrin (in each of five species for which sequence information is available), tenascin (in each of four species studied), laminin A (in mouse), and acetylcholinesterase (in Torpedo and human) (Jones et al., 1988; Nies et al., 1991; Nishi et al., 1991; Rupp et al., 1991, 1992; Sasaki et al., 1988; Schumacher et al., 1986; Smith et al., 1992; Soreq et al., 1990; Tsim et al., 1992; Weller et al., 1991).

The abundance of LRE-containing proteins in synaptic basal lamina and the ability of motoneuron-like cells to adhere to the LRE site in s-laminin suggest that LRE may provide a signal to motoneuronal growth cones when they reach synaptic sites. The experiments reported here were designed to define the role of LRE. We report that the LRE site in s-laminin inhibits neurite outgrowth from motoneuron-like cells and that growing neurites stop upon contacting an LRE-containing substrate. Our results support the idea that LRE is one of the components of the synaptic basal lamina that directs specific reinnervation at the neuromuscular junction.

Results

A Recombinant S-Laminin Fragment Inhibits Neurite Outgrowth

We showed previously that neurons from chick ciliary ganglia (CG), which innervate striated muscle in ovo (Tuttle, 1985), adhere to a carboxyl-terminal s-laminin fusion protein that includes the sequence LRE (Hunter et al., 1989b). We began the present study by extending the assay from <2 hr to >8 hr to ask how CG neurons were affected by contact with LRE. Though neurons adhered as well to the s-laminin fragment as to laminin, they extended neurites only on the latter. Although this result indicated only that the s-laminin fragment was unable to support neurite outgrowth, it raised the possibility that s-laminin could actually inhibit neurite outgrowth promoted by laminin. Because the fusion proteins used to study adhesion were soluble only in buffers that denatured laminin, we were unable to prepare mixtures of laminin and s-laminin with which to

*Present address: Klinikum der RWTH, Aachen, Germany.

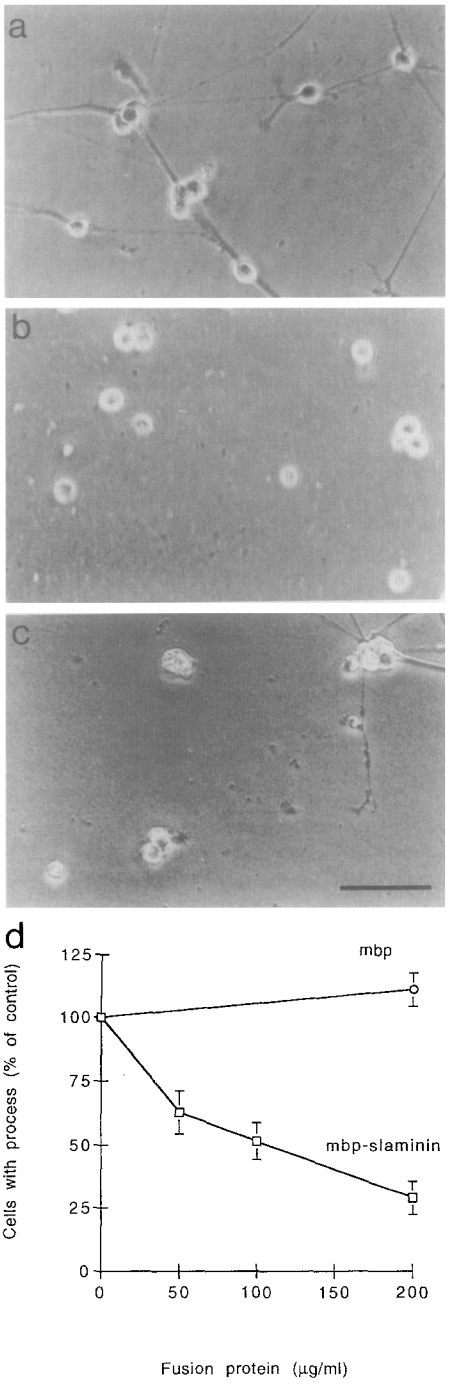

Figure 1. A C-Terminal Fragment of S-Laminin Inhibits the Neurite Outgrowth That Laminin Promotes

(a–c) Micrographs of CG neurons grown overnight on dishes coated with 20 μg/ml laminin (a), 100 μg/ml s-laminin–LRE (b), or a mixture of 20 μg/ml laminin and 100 μg/ml s-laminin–LRE (c). Bar, 5 μm.

(d) Dose-dependent inhibition of laminin-promoted neurite outgrowth by a s-laminin fragment fused to maltose-binding protein (mbp), but not by mbp alone. Values are mean ± SEM; n = 6.

test this idea. We therefore fused a large soluble protein, maltose-binding protein, to the same s-laminin sequence used for the adhesion assays. The resultant fusion protein, which we call s-laminin–LRE, was soluble in saline and could be mixed with laminin. When CG neurons were grown on tissue culture dishes coated with a mixture of laminin and s-laminin–LRE, fewer had neurites than when grown on laminin alone (Figures 1a–1c). This inhibition was specific, in that s-laminin–LRE inhibited outgrowth significantly at 50 μg/ml, whereas maltose-binding protein itself did not inhibit CG neurite outgrowth at concentrations up to 200 μg/ml (Figure 1d).

LRE Is Necessary and Sufficient for Inhibition

We took two approaches to determine whether the LRE site in the s-laminin–LRE fusion protein was responsible for the inhibition of CG neurite outgrowth. First, LRE was mutated to either Gln-Arg-Glu (QRE) or Leu-Arg-Ala (LRA) and the mutant s-laminin fusion proteins were assayed for their ability to inhibit neurite outgrowth. These substitutions were based on previous experiments showing that soluble LRE inhibits cell adhesion to s-laminin but that QRE and LRA do not (Hunter et al., 1991). S-laminin sequences that lacked the LRE site did not inhibit neurite outgrowth from CG neurons (Figure 2A). Moreover, neurites were longer, on average, on s-laminin–QRE than on s-laminin–LRE (140 ± 15 μm vs. 60 ± 10 μm, mean ± SEM, n = 200, at 16 hr in vitro). Thus, the LRE sequence is necessary for the s-laminin fusion protein to inhibit neurite outgrowth. In addition, the inactivity of the mutants provides further evidence that s-laminin–LRE does not inhibit neurite outgrowth by a nonspecific mechanism such as blocking the binding of laminin to the substratum.

The second approach was to generate small fusion proteins containing 4 or 8 copies of an LRE-containing decapeptide. These fusion proteins, 4 × LRE and 8 × LRE, inhibited neurite outgrowth at concentrations as low as 1 μg/ml (Figure 2B). A control fusion protein purified in the same manner did not inhibit neurite outgrowth. The 4 × LRE and 8 × LRE fusion proteins were ~50 times more inhibitory than s-laminin–LRE by weight. However, when the potency was calculated per mole of LRE, all three fusion proteins were inhibitory at approximately the same concentration (Figure 2C). Thus, the LRE site in s-laminin is not only necessary, but also sufficient for inhibiting CG neurite outgrowth.

Neurite Outgrowth Inhibition by LRE Is Restricted to Motoneuronal Cell Types

In previous studies, we showed that the LRE site in s-laminin was selectively adhesive for motoneuron-like cells. That is, CG neurons and NSC-34 cells, a putative spinal cord motoneuron-neuroblastoma hybrid, were adherent to s-laminin, whereas a variety of other cell types, including PC12 cells and dorsal root ganglion (DRG) neurons, were not (Hunter et al., 1989b, 1991). Here, we wanted to determine whether neurite outgrowth inhibition by LRE is similarly restricted. Experiments with mixed substrates carried out as described above showed that LRE inhibited neurite outgrowth from NSC-34 cells (Figure 3A), but not from PC12 cells (Figure 3B). S-laminin–LRE did inhibit outgrowth from DRG cells at high concentrations, but this inhibition was nonspecific in that it was also observed with the control fusion protein (Figure 3C). These

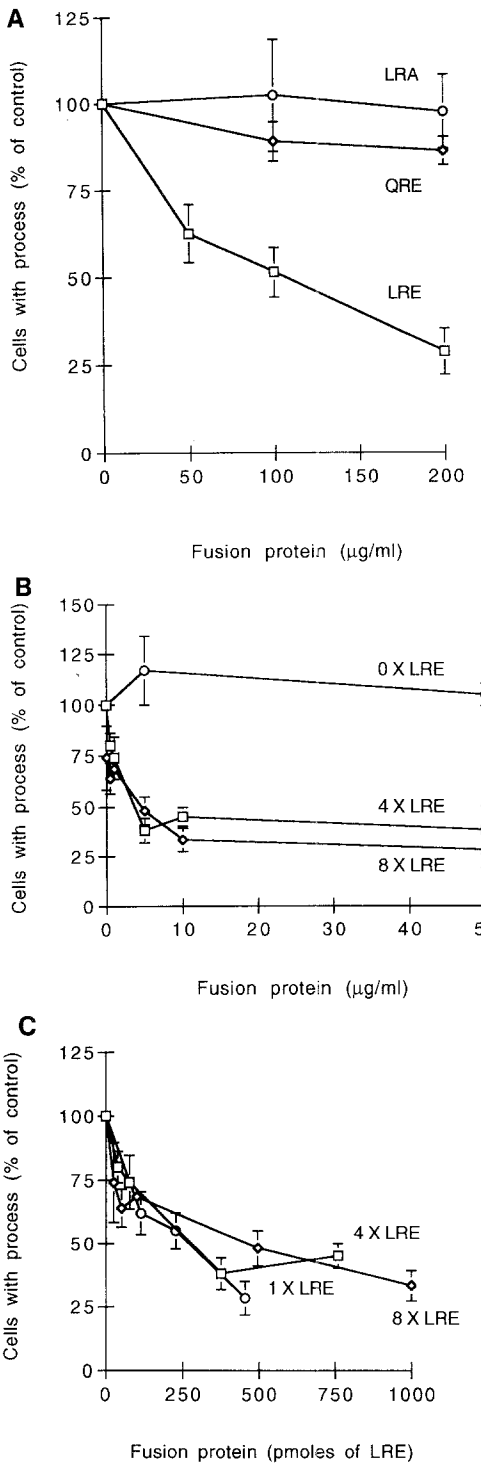

Figure 2. The LRE Site Is Necessary and Sufficient for S-Laminin to Inhibit Neurite Outgrowth

CG neurons were grown overnight on mixtures of 20 μg/ml laminin and indicated amounts of fusion proteins.

(A) Mutation of the LRE site in the recombinant s-laminin fragment to either QRE or LRA abolishes s-laminin inhibition of CG neurite outgrowth.

(B) CG neurite outgrowth is inhibited by fusion proteins containing 4 or 8 copies of an LRE-containing decapeptide.

(C) Data from (A) and (B) replotted as a function of LRE concentration. Values are mean ± SEM; n = 4–8.

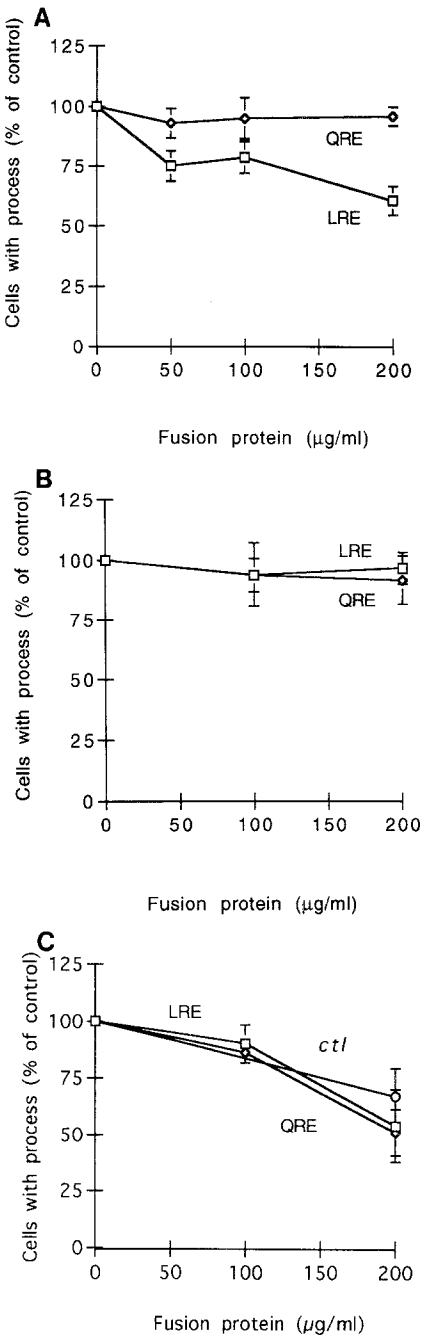

Figure 3. LRE Selectively Inhibits Neurite Outgrowth from Motoneuron-like Cells

NSC-34 cells (A), PC12 cells (B), or dorsal root ganglion cells (C) were grown overnight on mixtures of s-laminin fusion proteins and 20 μg/ml laminin. Only NSC-34 cells, which are motoneuron like, displayed LRE-dependent inhibition of laminin-promoted outgrowth. ctl, control protein (MBP). Values are mean ± SEM; n = 4–6.

results suggest that the same LRE-dependent mechanism is responsible for both cell adhesion and neurite outgrowth inhibition.

We also asked whether spinal motoneurons displayed LRE-dependent adhesion and inhibition of outgrowth. Cells were dissociated from E4–E5 chick spinal cords, and

a motoneuron-rich fraction was isolated by the method of Schnaar and Schaffner (1981) as modified by Dohrmann et al. (1986). The motoneurons adhered well to s-laminin–LRE (data not shown). Moreover, LRE-containing fusion proteins inhibited laminin-promoted outgrowth: the number of motoneurons with neurites was reduced by ~50% at 10 μg/ml 4 × LRE and by ~60% at 25 μg/ml s-laminin–LRE. To determine whether the inhibition was specific, we tested bacterially produced control proteins, such as maltose-binding protein (the s-laminin–LRE leader) or β-galactosidase. These failed to inhibit outgrowth detectably at concentrations of up to 50 μg/ml, or 2- to 5-fold higher than the LRE-containing proteins. Thus, spinal motoneurons display LRE-dependent inhibition of outgrowth. On the other hand, s-laminin–QRE also inhibited laminin-promoted outgrowth, although it was less than half as potent as s-laminin–LRE. This result raises the possibility that s-laminin–LRE contains a second site that motoneurons recognize. Additional studies to test this idea are now in progress.

LRE Inhibits Neurite Outgrowth on Laminin, Fibronectin, and Collagen I but Not on Collagen IV

The experiments described so far used substrates in which s-laminin-derived fragments were mixed with laminin. We next tested the ability of LRE to inhibit neurite outgrowth promoted by other substrates. It was not feasible to use CG neurons for this purpose, because in our cultures they extended neurites poorly on substrates other than laminin. In contrast, NSC-34 cells extended neurites that appeared similar on laminin, collagen I, and collagen IV. Fibronectin also induced substantial neurite outgrowth from the NSC-34 cells but caused cell bodies to flatten as well. To assure that any inhibition detected was not an artifact of the method used to prepare the substrate, we performed these assays in two ways. First, we mixed each substrate with either s-laminin–LRE or s-laminin–QRE and compared neurite outgrowth on the two mixtures. Second, we compared outgrowth on layered substrates composed of an outgrowth-promoting material atop either active or inactive s-laminin–LRE. To inactivate s-laminin–LRE, we used ultraviolet (UV) irradiation, which has been shown by others to inactivate substrate-bound laminin and tenascin (Hammarback et al., 1985; Faissner and Kruse, 1990). The s-laminin–LRE was then overlayed with laminin, fibronectin, collagen I, or collagen IV.

The two assays gave consistent results. When tested as a mixture, s-laminin–LRE inhibited neurite outgrowth promoted by laminin, fibronectin, or collagen I, but not by collagen IV (Figure 4A). Similarly, active but not UV-inactivated s-laminin–LRE inhibited neurite outgrowth when overlaid with laminin, fibronectin, and collagen I, whereas neurite outgrowth was similar on active and inactive s-laminin overlaid with collagen IV (Figure 4B). These data suggest that s-laminin–LRE does not inhibit outgrowth by interfering with a specific neurite outgrowth-promoting site on laminin; instead it inhibits by a general, but not universal, mechanism.

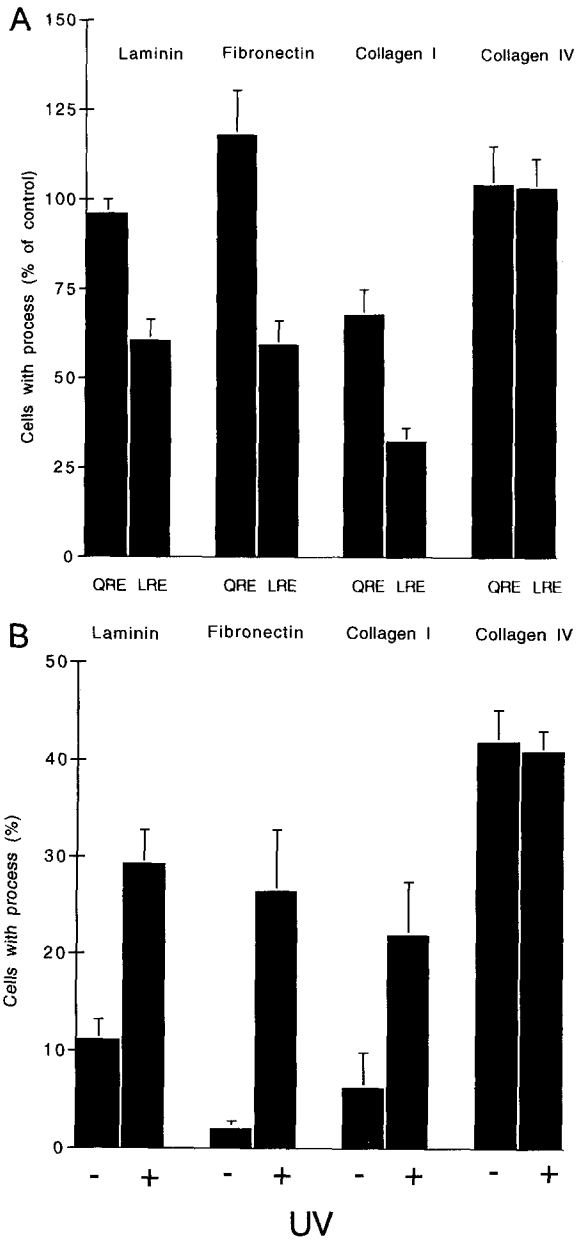

Figure 4. S-Laminin Inhibits Neurite Outgrowth Promoted by Laminin, Fibronectin, and Collagen Type I but Not by Collagen Type IV

(A) NSC-34 cells were grown for 2 hr on mixtures of s-laminin–LRE or s-laminin–QRE (200 μg/ml) and the indicated matrix protein (20 μg/ml).

(B) NSC-34 cells were grown on active or UV-inactivated s-laminin–LRE overlaid with matrix proteins (20–40 μg/ml). Values are mean ± SD; n = 4–12.

Neurites Do Not Extend onto Regions Containing S-Laminin–LRE

In the experiments presented so far, neurons were exposed to uniform substrates containing mixtures of outgrowth-promoting and outgrowth-inhibiting materials. In vivo, in contrast, it is only the terminal portion of a regener-

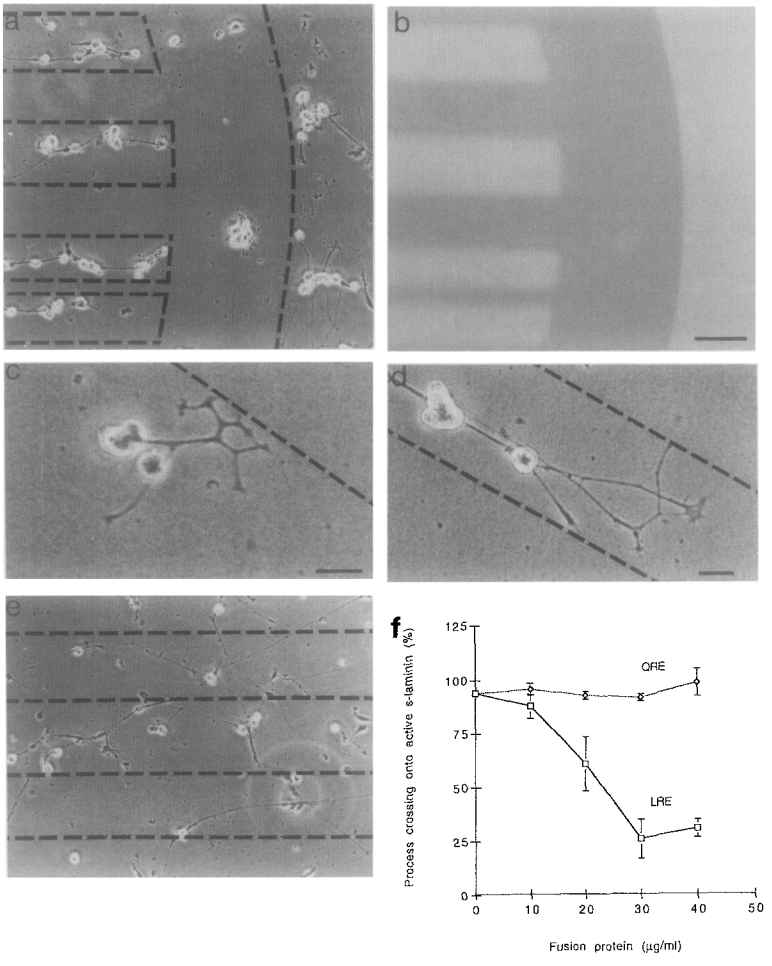

Figure 5. Neurites Seldom Grow onto S-Laminin–LRE-Containing Substrates

CG neurons were grown overnight on patterned substrates prepared as described in the text, then viewed under brightfield (a and c) or epifluorescence (b) optics. The dark regions in (b) were shielded from UV irradiation and therefore contain active s-laminin. The entire surface is overlaid with laminin. Few neurites cross onto active s-laminin–LRE (a and b). Higher power micrographs of neurites at borders are shown in (c and d). Neurites cross readily onto s-laminin–QRE (e). Borders seen on epifluorescence migrographs were drawn onto the phase micrographs on (a) and (c–e). Compilation of data from three experiments is shown in (f). Bar, 50 μm (a, b, and e); 20 μm (c and d).

ating motor axon that contacts the LRE-enriched synaptic basal lamina. To model this situation, we produced patterned substrates with alternating stripes of active and inactive s-laminin on a homogeneous layer of laminin. The method we used is based on the UV inactivation protocol described above (see Figure 4B). A dish was coated with s-laminin fusion protein, a grid was placed on top of the substrate, and the dish was exposed to UV light. Then the grid was removed and the dish was coated with laminin. This protocol generates alternating stripes of inactivated (UV-irradiated) and active (shielded by the grid) s-laminin, all overlaid by a uniform layer of laminin. CG neurons were grown overnight on these patterned substrates, then examined by phase microscopy. Neurites growing on laminin plus inactive s-laminin–LRE seldom crossed onto laminin plus active s-laminin–LRE (Figures 5a–5d). Inhibition was dose dependent (Figure 5f). In contrast, neurites grew equally well on active or inactive s-laminin–QRE and crossed randomly onto regions of active s-laminin–QRE (Figures 5e and 5f). As above, the use of s-laminin–QRE demonstrates that the inhibition is LRE-dependent and rules out nonspecific toxic effects of the UV treatment or the grid. Thus, neurites avoid regions of substrate that contain s-laminin–LRE.

Growth Cones Stop at LRE-Containing Borders

The experiments illustrated in Figure 5 showed that CG neurites recognized s-laminin–LRE-containing borders but did not reveal what growth cones did when they contacted a border. They might, for example, turn, stop, or collapse (see Discussion). To distinguish among these and other possibilities, we used time-lapse video microscopy to monitor neurites at the laminin/s-laminin–LRE borders. The trajectories of 36 neurites that contacted s-laminin–LRE borders were reconstructed. Consistent with the prior observation (Figure 5d) that ~30% of neurites crossed onto the s-laminin–LRE substrate, 13 of the neurites grew past the border. The other 20 neurites stopped growing when they contacted the border. One example is shown in Figure 6. Here, a neuron bore no processes when viewed initially, but extended a neurite that contacted the border within the first hour. Over the next 9 hr, the growth cone slid along the border somewhat, but did not advance, retract, or turn appreciably. Then, over a 1 hr period, the neurite retracted toward the cell body. Overall, periods of growth arrest ranged from 0.5 to 18 hr (Figure 7); the average was 3.8 hr. A few neurites in addition to the one shown in Figure 6 slid along the border during the period of arrest, but most were appar-

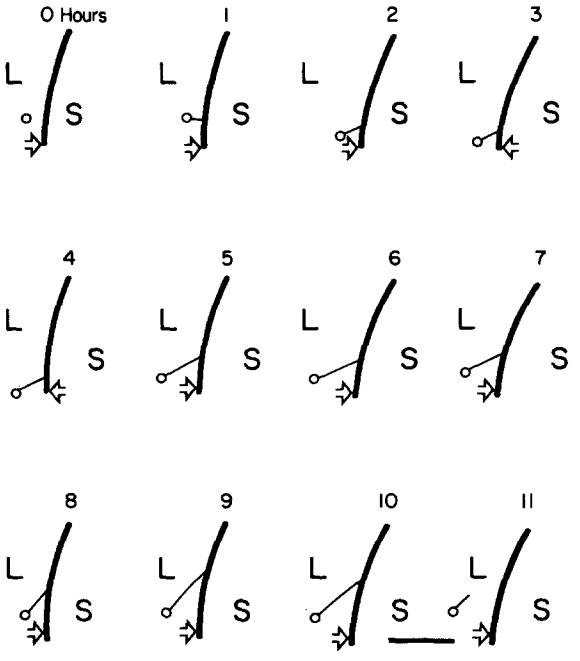

Figure 6. Neurites Stop at the Edge of an S-Laminin–LRE-Containing Substrate

Sketches of a single CG neuron taken at 1 hr intervals from a 12 hr time-lapse video record. The growth cone extended on laminin (L), then stopped upon reaching the edge of the s-laminin–LRE-containing substrate (S). The neurite remained at the border for ~10 hr before retracting. Arrow shows position of an imperfection on the substrate used as a landmark. Bar, 100 μm.

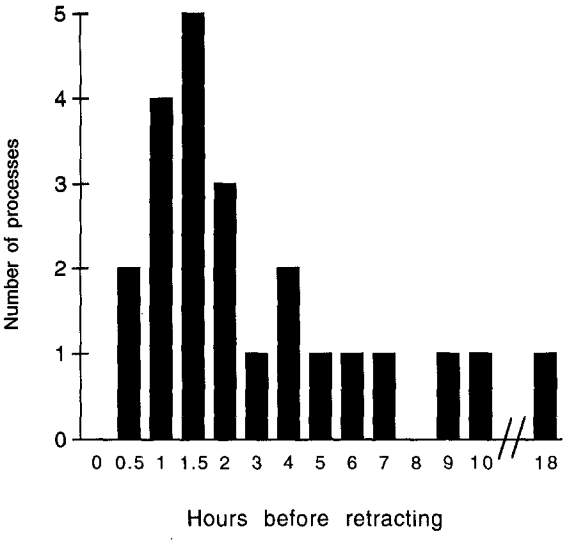

Figure 7. Growth Arrest of Neurites at a S-Laminin–LRE Border

Thirty-six CG neurites contacting the edges of s-laminin–LRE substrates were viewed for up to 24 hr. Thirteen crossed the border without a detectable pause, and none retracted within 0.5 hr following contact. The length of time the remaining twenty-three growth cones remained at the border is plotted. All of these neurites eventually retracted away from the border.

ently immobile. All but 1 of the 20 neurites then retracted; the remaining neurite eventually turned and grew away from the border. The retraction was rapid, with an entire neurite sometimes disappearing in the interval between successive images (15 min). Thus, LRE appears to act as a stop signal for growing neurites. After prolonged exposure, it may also contribute to neurite retraction. In our cultures, however, neurites that were not contacting a border often collapsed as well, so we do not know whether contact with the active s-laminin–LRE substrate actually led to neurite retraction.

Discussion

We showed previously that s-laminin is concentrated in the basal lamina of the neuromuscular junction and that it contains a binding site that is selectively adhesive for motoneuron-like cells (Hunter et al., 1989b, 1991). These observations suggested that s-laminin might comprise part of a signal that motor neuronal growth cones encounter at synaptic sites. Accordingly, we undertook a study to learn how binding to a s-laminin fragment affected the behavior of motor axons. We used ciliary neurons in most experiments because they are motoneurons—they innervate striated muscle in vivo as well as in vitro—but are easier to isolate and maintain in culture than spinal motoneurons (see, for example, Bixby and Reichardt, 1985; Role et al., 1985; Lupa et al., 1990). In addition, we tested chick spinal motoneurons and a mouse motoneuron-like cell line, NSC-34. We show here that s-laminin inhibits neurite outgrowth promoted by other matrix molecules. LRE is necessary and sufficient for this inhibition, and the inhibition is exhibited only by neurons that adhere to LRE. Moreover, at least one substrate is immune to the inhibitory effect of LRE, suggesting that outgrowth inhibition is context dependent. Finally, we show that neurites stop growing upon encountering a local accumulation of LRE, a behavior that may be related to that exhibited by regenerating motor axons in vivo.

LRE as an Inhibitory Signal

It is remarkably easy to inhibit outgrowth from cultured neurons by nonspecific means. For example, a putative inhibitory factor might be generally toxic for neurons, or it might simply prevent an outgrowth promoter from binding to the tissue culture dish. The simple observation that neurites grow poorly in the presence of a s-laminin fragment is therefore of little significance. Our confidence that the inhibitory effect of s-laminin is specific is based on several lines of evidence (see Figure 2, Figure 3, and Figure 4). First, s-laminin–LRE inhibited laminin-promoted outgrowth, but s-laminin–QRE and s-laminin–LRA did not. These three fusion proteins differed only in a single amino acid and were prepared and purified in the same way. It is highly unlikely that one of them would have been nonspecifically toxic in a way that the others were not. Second, s-laminin LRE inhibited outgrowth not only when it was mixed with laminin but also when it was applied to the substrate before laminin was added. It is therefore unlikely that it covered a promoting site on laminin or sterically hindered neuronal access to laminin in a nonspecific way. Third, the inhibitory effect of s-laminin–LRE was abolished

by UV irradiation. This result provides assurance that the fusion protein was not merely preventing the binding of laminin to the substrate. Finally, the observation that s-laminin–LRE inhibited outgrowth promoted by several but not all substrates indicates that the effect reflected neither a peculiarity of laminin-promoted outgrowth nor a general attenuation of neuronal health. Instead, we conclude that s-laminin–LRE inhibits neurite outgrowth by a mechanism that involves specific binding to a cellular receptor.

Three lines of evidence point to the LRE tripeptide as being an important component of the binding site for this putative receptor. First, cell types that display LRE-dependent adhesion (CG neurons and NSC-34 cells) are inhibited by s-laminin–LRE, whereas cells that do not adhere to s-laminin–LRE (PC12 cells and sensory neurons) are not inhibited by it. Second, single amino acid changes in the LRE sequence of the s-laminin–LRE fragment to either QRE or LRA eliminated the inhibitory activity. Third, the 4 × LRE and 8 × LRE fusion proteins, composed of 4 or 8 LRE-containing decapeptide repeats, were ~50-fold more inhibitory than s-laminin–LRE by weight but equally active when compared per mole of LRE. Thus, the LRE site appears to account completely for the inhibition of CG neurite outgrowth by the s-laminin fragment.

How might LRE inhibit neurite outgrowth? Some neurite outgrowth inhibitors act by complexing with an active site on an outgrowth promoter (Muir et al., 1989), but evidence summarized above indicates that the LRE receptor is on the cell, not the substrate. Alternatively, s-laminin–LRE could inhibit neurite outgrowth by decreasing neuronal adhesivity to the substrate. Several proteoglycans and members of the tenascin family inhibit both neuronal adhesion and neurite outgrowth, suggesting that poor adhesion can prevent neurite outgrowth (Pesheva et al., 1989; Faissner and Kruse, 1990; Cole and McCabe, 1991). This explanation is unlikely to account for the inhibition we observed, however, because s-laminin–LRE promotes rather than inhibits adhesion. Similarly, s-laminin–LRE is not appreciably more adhesive for neurons than laminin, arguing that LRE does not "glue" neurites to the substrate so tightly that they cannot extend. Our results are consistent with a growing body of evidence showing that adhesive strength correlates poorly with neurite outgrowth (Letourneau, 1975; Gundersen, 1987; Lemmon et al., 1992). Instead, we suspect that occupancy of an LRE receptor on the cell surface activates second messenger systems that block neurite outgrowth (Schwab et al., 1993).

Consistent with this idea is the finding that s-laminin–LRE inhibited NSC-34 neurite outgrowth on several substrates but not on collagen IV. Numerous intracellular pathways appear to be involved in inducing neurite outgrowth (Bixby and Harris, 1991; Bixby, 1992), and an inhibitory molecule might block only a subset of them. We speculate that collagen IV promotes neurite outgrowth through a mechanism that either overwhelms or circumvents the effect of LRE. It will be important to seek second messenger pathways that mediate LRE-dependent inhibition; so far, only a few such studies have been reported for any inhibitors of outgrowth (Igarashi et al., 1993; Bandtlow et al., 1993; Schwab et al., 1993). Whatever the mechanism, however, our data suggest that the context in which the s-laminin molecule is expressed will influence its activity.

LRE as a Stop Signal

Experiments in which neurons were plated on uniform substrates demonstrated LRE-dependent inhibition of neurite outgrowth but could not distinguish between an effect on initiation and an effect on elongation. This distinction is important, because it is the growth cones of axons extending on a growth-promoting substance that encounter s-laminin at the neuromuscular junction in vivo. We therefore turned to patterned substrates, modifying the method of Hammerback et al. (1985) to prepare alternating stripes of active and inactivated s-laminin–LRE or s-laminin–QRE overlaid with a uniform coat of laminin. We adopted this somewhat complex protocol to ensure that observed effects could be ascribed to LRE-dependent interactions rather than to an unintended depletion of the growth-promoting substrate by the putative inhibitory material.

Using this method, we found that neurites growing on laminin failed to extend onto active s-laminin–LRE, whereas they extended without impediment onto s-laminin–QRE (see Figure 5). Thus, growth cones, as well as cell bodies, are likely to bear LRE receptors. More importantly, time-lapse video microscopy demonstrated that the growth cones stopped growing at the borders (see Figure 6 and Figure 7). This behavior differs from that reported for neurites encountering several growth-inhibitory components that are generally considered to be components of axonal pathways: tectal membranes (Walter et al., 1987), chondroitin sulfate proteoglycan (Snow et al., 1991), tenascin, and januscin/restrictin (Taylor et al., 1993). In those cases, growth cones sometimes slowed their rate of growth but then generally turned and grew along the border. As noted by Baird et al. (1992b), it might be expected that outgrowth inhibition along an axonal pathway would result in transient arrest and subsequent redirection, whereas inhibition at a target would result in longer-lasting arrest and eventual synaptic differentiation.

Whether these different consequences of inhibition reflect different mechanisms remains unclear. In several cases, factors that lead to axonal rerouting act by causing growth cones to retract or collapse (Kapfhammer and Raper, 1987; Cox et al., 1990; Luo et al., 1993; Bandtlow et al., 1993). Following collapse, the growth cone reextends and sets off in a new direction. We have not observed growth cones to collapse either directly after contacting a s-laminin–LRE border or when challenged with LRE in solution (B. E. P., unpublished data). Indeed, when we observed growth cones at high magnification in fixed cultures, we frequently saw large lamellopodia rather than the compact appearance typical of retracting processes (see Figures 5c and 5d). Moreover, the time course of the retraction that eventually occurred was much slower than that reported for collapse. On the other hand, both Bandtlow et al. (1990) and Oakley and Tosney (1993) have described target-derived signals that apparently lead to rapid collapse in one context but stable arrest in another. It will be important to learn whether the arrest we have docu-

mented is a fundamental consequence of activating an LRE-receptor, or whether the response to s-laminin–LRE depends on how it is presented.

LRE at the Neuromuscular Junction

This study was based on the speculation that s-laminin, and particularly its LRE site, might serve as a muscle-derived retrograde signal for motor axons at the neuromuscular junction. Three points are worth mentioning in this context.

First, a prominent feature of synaptogenesis is that growth cones contacting postsynaptic surfaces stop growing and start to differentiate. During reinnervation of the neuromuscular junction, some branches sprout beyond s-laminin-rich original synaptic sites, but the majority stop there and differentiate into nerve terminals. The notion that s-laminin provides a stop signal thus provides a partial explanation for the phenomenon of selective reinnervation of original synaptic sites (Hall and Sanes, 1993). Differentiation, then, might be a consequence of stopping, a separate LRE-mediated effect, or the response to a different retrograde signal. The possibility that s-laminin itself might be involved in terminal differentiation is raised by recent observations of a s-laminin-deficient mutant mouse; differentiation of motor nerve terminals is clearly aberrant in these mice (P. G. Noakes et al., unpublished data). Based on these results, we are currently asking whether axons that contact s-laminin–LRE in vitro acquire any features of differentiated nerve terminals.

Second, the cell-type specificity of inhibition documented in vitro correlates with a cellular selectivity observed in vivo. That is, nerves that supply muscles contain as many sensory and sympathetic axons as motor axons, yet only the motor axons differentiate into nerve terminals at synaptic sites on muscle fibers (e.g., Ochí et al., 1992). Correspondingly, outgrowth from motoneuron-like cells is inhibited by s-laminin–LRE, whereas outgrowth from sensory neurons and NGF-treated PC12 cells (which resemble sympathetic neurons) is not. Moreover, within the CG, which we used here as a source of motoneurons, the ciliary neurons, which normally innervate striated muscle, display LRE-dependent adhesion, whereas the choroid neurons, which normally innervate smooth muscle (Tuttle, 1985) do not (B. E. P and J. R. S., unpublished data). In fact, it is quite likely that the presence of these distinct LRE-sensitive and LRE-insensitive populations within the CG, rather than limited efficacy of the s-laminin–LRE, accounts for the partial inhibition apparent in our experiments (e.g., see Figure 1c, Figure 2c, and Figure 5d).

Finally, the observation that s-laminin–LRE does not inhibit neurite outgrowth promoted by collagen IV is noteworthy, in that collagen IV is a prominent component of most basal laminae. In this context, it is intriguing that synaptic basal lamina contains very low levels of the α1(IV) and α2(IV) chains, which are the predominant chains in most tissues and the chains that were available for us to test. Instead, the synapse is rich in the α3, α4, and α5 chains of collagen IV (Sanes et al., 1990; Miner and Sanes, 1994). It is possible that this synaptic specialization in collagen IV chains could potentiate the effects of s-laminin–LRE, and it will therefore be interesting to test the effects of the α3–α5(IV) chains on neurite outgrowth, both alone and in the presence of s-laminin.

Experimental Procedures

Fusion Proteins

A 677 bp C-terminal s-laminin cDNA fragment, (RK 36; Hunter et al., 1989b) was subcloned into M13 and grown in JM109 bacteria. Site-directed mutagenesis was performed by the method of Burke and Olson (1986). In brief, single-stranded phage DNA was incubated with 21 bp oligonucleotides that encoded the mutated sequences. After 10 min at 70°C, the DNA was transformed into bacteria, resulting phages were grown in JM109, and plaques were screened with ^{32}P-labeled oligonucleotide probes. The mutations were confirmed by DNA sequencing of the entire insert. Wild-type and mutant s-laminin fragments were subcloned into the EcoRI site of Pmal-Cr2 (New England Biolabs, Beverly, MA) to generate a fusion of maltose-binding protein and the s-laminin fragment. The constructs were transformed into a *lon* protease-deficient strain of bacteria (SW6AA2; New England Biolabs). The bacterial supernatant was purified on maltose–agarose as described by Riggs (1990), and column fractions were analyzed by gel electrophoresis and immunoblotting. The eluates contained a major species that had the expected size, ~66 kDa, and was recognized by both anti-maltose-binding protein antibody (New England Biolabs) and anti-s-laminin antibody D5 (Hunter et al., 1989b) on Western blots.

Fusion proteins containing 4 or 8 copies of LRE were generated from concatamers of synthetic oligonucleotides. Two partially complementary 30 bp oligonucleotides were synthesized, 5′AGCTTCGAGAACAAGTAGGAGACCAAAAGC3′ and 5′AGCTGCTTTTGGTCTCCTACTGTTCTCGA3′. The first oligonucleotide was phosphorylated on the 5′-end using a 5′ Phosphate-On kit (Clonetech, Palo Alto, CA). The oligonucleotides were annealed to produce a duplex that contained complementary 4 bp overhangs and encoded an s-laminin-derived decapeptide, LREQVGDQKQ. The annealed oligonucleotides were ligated with T4 ligase. Because only one strand is phosphorylated, the ligated concatamers were expected to be directed repeats. A fragment containing four tandem repeats was introduced into the HindIII site of the pET 23b expression vector (Novagen, Madison, WI), which encodes a 4 kDa leader and a short carboxyl-terminal extension that includes a polyhistidine sequence. The polyhistidine tag binds to nickel and is used to purify the fusion protein from bacterial lysate. Following transfection into DE3 bacteria, a nickel-binding protein of the expected size, ~8 kDa, was expressed. For use in adhesion assays, the protein was purified to apparent homogeneity on a nickel-charged column (HIS Bind; Novagen) according to the instructions of the manufacturer. The structure of the oligomer was confirmed by sequence analysis of the plasmid and amino acid analysis of the purified protein.

To produce 8 copies of the LRE-containing decapeptide, the SalI to XhoI fragment containing the 4 LRE repeats was subcloned into the XhoI site of 4 × LRE. Plasmids containing the insert in the correct orientation were used to express a ~12 kDa protein that was purified as described above. β-galactosidase fusion protein (Novagen) was also purified as described above. Eluates were dialyzed against phosphate-buffered saline (PBS) overnight before use.

Tissue Culture

CG were dissected from E8-E9 chick embryos (SPAFAS, Roanoke IL) and dissociated in 0.1% trypsin (Porcine type IX; Sigma, St. Louis, MO) for 15 min at 37°C. Cells were triturated and then plated in minimal essential medium (MEM; Gibco, Gaithersburg, MD) containing 10 mM HEPES, 5% chicken embryo extract, 10% fetal calf serum (FCS), 10 U/ml penicillin, and 10 μg/ml streptomycin. Approximately ⅓ ganglion was plated per well of a 24 well dish. Incubation was at 37°C in 7% CO_2. **DRG** were dissected from E7-E8 chickens and dissociated as described for ciliary cells. Culture medium was a 1:1 mixture of Dulbecos' modified Eagle's medium (DMEM) and F12 (Gibco) with 1% FCS, 10 U/ml penicillin, 10 μg/ml streptomycin, and 50 ng/ml of NGF (generously provided by P. Osborne and E. Johnson, Washington University). **PC12** cells were primed prior to assay by growing them for 7 days on

dishes coated with collagen I (Vitrogen; Celltrix, Palo Alto, CA) in RPMI 1640 containing 10% heat inactivated horse serum, 5% FCS, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg TML streptomycin, and 50 ng/ml NGF (Greene et al., 1982). Media was changed to RPMI 1640, 1% horse serum, 1 mM EGTA, and 50 ng/ml NGF the night prior to the outgrowth assay to minimize cell clumping. On the day of the assay, cells were washed three times in 50% RPMI 1640 (1:1; Greene et al., 1987), then replated in the same medium. **NSC-34** cells (Cashman et al., 1992) were grown in DMEM containing 3% FCS. For the outgrowth assay, the cells were seeded at approximately 5×10^3 cells per well in calcium-free Hank's balanced salt solution with 0.8 mM $MgCl_2$ and 10 mg/ml bovine serum albumin. **Spinal motoneurons** were isolated from spinal cords of E4–E5 chick embryos by the method of Schnaar and Schaffner (1981) as modified by Dohrmann et al. (1986). In brief, spinal cords were dissociated in 0.5% trypsin plus 10 μg/ml DNaseI (Sigma) for 15 min at 37°C. Cells were resuspended, triturated, and layered on a 6.8% metrizamide (Sigma) cushion. Following centrifugation for 15 min at 520 × g, cells at the interface were removed, diluted in medium, recentrifuged, and resuspended in growth medium, which was DMEM supplemented with 10% FCS, 2.5% chick embryo extract, 5% chick muscle extract, 2 mM glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin.

Neurite Outgrowth

For the experiments with CG, DRG, PC12 cells, and spinal motoneurons, 24 well dishes were coated with methanol-solubilized nitrocellulose (BA85; Schleicher and Schuell, Keene, NH) as described by Lagenauer and Lemmon (1987). Fusion protein was mixed with laminin (20 μg/ml final concentration; Collaborative Biomedical Products, Bedford, MA), and 150 μl of the mixture was applied per well. After a 2 hr incubation, the plate was blocked with 30 mg/ml bovine serum albumin in PBS.

To prepare substrates for NSC-34 cells, fusion protein was mixed with laminin, fibronectin (Sigma), collagen IV (Collaborative Biomedical Products), or collagen I (all at 20 μg/ml), and 1.5 μl spots were applied to nitrocellulose coated wells in 12 well plates. After 1 hr, drops were aspirated and the wells were washed with 75 μg/ml keyhole limpet hemocyanin (Sigma) in PBS blocked for one hour with 0.75 mg/ml keyhole limpet hemocyanin, and washed with PBS. Alternatively, spots of pET 36 fusion protein (30 μg/ml; Hunter et al., 1989b) were applied for 20 min, aspirated, and washed with PBS. Some of the wells were then exposed to UV light, either for 20 min in a Stratalinker 2400 (Stratagene, La Jolla, CA) or for 1.5 hr using a UV light box (Model UVB-15E; Ultralum, Carson, CA). Subsequently, the wells were overlaid with laminin, collagen IV, fibronectin, or collagen I at 20 μg/ml for 1 hr, then blocked and washed as described above.

For neurite outgrowth analysis, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. Each well was examined by counting all the cells in a horizontal strip of 20× microscopic fields across the well. A cell was scored as bearing a neurite if the length of any process was greater than two times the diameter of the cell body. Neurite lengths were measured with a microcomputer-based image analysis system (Image 1; Universal Imaging Corporation, Westchester, PA). Outgrowth was routinely analyzed after ~16 hr for CG, PC12, and DRG cells. Motoneurons were analyzed after 24–72 hr because they exhibited a substantial lag before initiating outgrowth. NSC-34 cells were assayed 2 hr after plating because they initiated outgrowth within 30 min of plating, and their processes reached maximal length in <4 hr. NSC-34 cells never extended neurites on s-laminin–LRE, even when they were maintained on this substrate for 24 hr.

Patterned Substrates

Patterned substrates were produced by a method similar to that described by Hammerback et al. (1985). Wells in a 12 well dish were coated with nitrocellulose, then incubated for 1 hr with 150 μl of fusion protein, washed with PBS, and air dried. Nickel parallel bar or Sjostrand electron microscopy grids (EM Sciences, Fort Washington, PA) were distributed around the wells, and the plate was exposed to UV light as described above. The wells were then washed with PBS, coated with 200 μl of 20-40 μg/ml of laminin for 2–3 hr, and washed again just before adding cells.

CG cultures were fixed in 4% paraformaldehyde and analyzed on an inverted microscope with a combination of phase and epifluorescence optics. Tissue culture plastic autofluoresces following exposure to UV light, so the area initially covered by the grid was later visible as a dark nonfluorescent area. We suspect that the UV light interacts with the tissue culture plastic because nitrocellulose-coated glass coverslips tested in a similar manner did not fluoresce. When treated wells were viewed with phase optics, no border was visible, suggesting that the electron microscopy grid did not leave a physical barrier to neurite outgrowth. For video imaging, cultures were first placed in a 7% CO_2 incubator for approximately 2 hr to allow the cells to adhere and the media to equilibrate to pH 7.4. The tissue culture well was sealed with silicon grease and maintained at 35°C in a heated tent encasing a Nikon diaphot microscope. Images were taken at 10× magnification using an MTI low light camera (Dage-MTI Inc., Michigan City, IN). At 15 min intervals, a timing device opened a shutter, exposing the cells to light and simultaneously activating a Panasonic Optical Disk Recorder-TQ2028F (Panasonic Industrial Division, Secaucus, NJ). At the end of an experiment, the plate was photographed under UV illumination to mark the placement of the electron microscopy grid.

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References

Baird, D. H., Hatten, M. E., and Mason, C. A. (1992a). Cerebellar target neurons provide a stop signal for afferent neurite extension in vitro. J. Neurosci. *12*, 619–634.

Baird, D. H., Baptista, C. A., Wang, L. C., and Mason, C. A. (1992b). Specificity of a target cell-derived stop signal for afferent axonal growth. J. Neurobiol. *23*, 579–591.

Bandtlow, C., Zachleder, T., and Schwab, M. E. (1990). Oligodendrocytes arrest neurite growth by contact inhibition. J. Neurosci. *10*, 3837–3848.

Bandtlow, C. E., Schmidt, M. F., Hassinger, T. D., Schwab, M. E., and Kater, S. B. (1993). Role of intracellular calcium in NI-35 evoked collapse of neuronal growth cones. Science *259*, 80–83.

Bastmeyer, M., and Stuermer, C. A. (1993). Behavior of fish retinal growth cones encountering chick caudal tectal membranes: a time-lapse study on growth cone collapse. J. Neurobiol. *24*, 37–50.

Bixby, J. L. (1992). Diversity of axonal growth-promoting receptors and regulation of their function. Curr. Opin. Neurobiol. *2*, 66–69.

Bixby, J. L., and Reichardt, L. F. (1985). The expression and localization of synaptic vesicle antigens at neuromuscular junctions in vitro. J. Neurosci. *5*, 3070–3080.

Bixby, J. L., and Harris, W. A. (1991). Molecular mechanisms of axon growth and guidance. Annu. Rev. Cell Biol. *7*, 117–159.

Buchanan, J., Sun, Y. A., and Poo, M. M. (1989). Studies of nerve-muscle interactions in Xenopus cell culture: fine structure of early functional contacts. J. Neurosci. *9*, 1540–1554.

Burgeson, R. E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H., Martin, G. R., Meneguzzi, G., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., and Yurchenco, P. D. (1994). A new nomenclature for the laminins. Matrix Biol. *14*, 209–211.

Burke, D. T., and Olson, M. V. (1986). Oligodeoxynucleotide-directed

mutagenesis of *Escherichia coli* and yeast by simple cotransformation of the primer template. DNA (NY). *5*, 325–332.

Cashman, N. R., Durham, H. D., Blussztajn, J. K., Oda, K., Tabira, T., Shaw, I. T., Dahrouge, S., and Antel, J. P. (1992). Neuroblastoma × spinal cord (NSC) hybrid cell lines resemble developing motor neurons. Dev. Dyn. *194*, 209–221.

Cole, G. J., and McCabe, C. F. (1991). Identification of a developmentally regulated keratan sulfate proteoglycan that inhibits cell adhesion and neurite outgrowth. Neuron *7*, 1007–1018.

Connor, E. A., and Smith, M. A. (1994). Retrograde signaling in the formation and maintenance of the neuromuscular junction. J. Neurobiol. *25*, 722–739.

Cooper, M. W., and Smith, S. J. (1992). A real-time analysis of growth cone-target cell interactions during the formation of stable contacts between hippocampal neurons in culture. J. Neurobiol. *23*, 814–828.

Cox, E. C., Muller, B., and Bonhoeffer, F. (1990). Axonal guidance in the chick visual system: posterior tectal membranes induce collapse of growth cones from temporal retina. Neuron *4*, 31–37.

Dai, Z., and Peng, H. B. (1993). Elevation in presynaptic Ca^{2+} level accompanying initial nerve–muscle contact in tissue culture. Neuron *10*, 827–837.

Dailey, M. E., and Smith, S. J. (1993). Confocal imaging of mossy fiber growth in live hippocampal slices. Jpn. J. Physiol. *43*, 183–192.

Dan, Y., and Poo, M.-M. (1994). Retrograde interactions during formation and elimination of neuromuscular synapses. Curr. Opin. Neurobiol. *4*, 95–100.

Dohrmann, U., Edgar, D., Sendtner, M., and Thoenen, H. (1986). Muscle-derived factors that support survival and promote fiber outgrowth from embryonic chick spinal motor neurons in culture. Dev. Biol. *118*, 209–221.

Faissner, A., and Kruse, J. (1990). J1/tenascin is a repulsive substrate for central nervous system neurons. Neuron *5*, 627–637.

Funte, L. R., and Haydon, P. G. (1993). Synaptic target contact enhances presynaptic calcium influx by activating cAMP-dependent protein kinase during synaptogenesis. Neuron *10*, 1069–1078.

Glicksman, M., and Sanes, J. R. (1983). Development of motor nerve terminals formed in the absence of muscles fibers. J. Neurocytol. *12*, 661–674.

Greene, L. A., Burstein, D. E., and Black, M. M. (1982). The role of transcription-dependent priming in nerve growth factor promoted neurite outgrowth. Dev. Biol. *91*, 305–316.

Greene, L. A., Aletta, J. M., Rukenstein, A., and Green, S. H. (1987). PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. Meth. Enzymol. *147*, 207–216.

Gundersen, R. W. (1987). Response of sensory growth cones to patterned substrata of laminin and fibronectin *in vitro*. Dev. Biol. *121*, 423–431.

Hall, Z. W., and Sanes, J. R. (1993). Synaptic structure and development: the neuromuscular junction. Cell *72*/Neuron *10* (Suppl.), 99–121.

Hammarback, J. A., Palm, S. L., Furcht, L. T., and Letourneau, P. C. (1985). Guidance of neurite outgrowth by pathways of substratum-absorbed laminin. J. Neurosci. Res. *13*, 213–220.

Hunter, D. D., Shah, V., Merlie, J. P., and Sanes, J. R. (1989a). A laminin like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. Nature *338*, 229–234.

Hunter, D. D., Porter, B. E., Bullock, J. W., Adams, S. P., Merlie, J. P., and Sanes, J. R. (1989b). Primary sequence of a motor neuron-selective adhesive site in the synaptic basal lamina protein s-laminin. Cell *59*, 905–913.

Hunter, D. D., Cashman, N., Morris-Valero, R., Bulock, J. W., Adams, S. P., and Sanes, J. R. (1991). An LRE (leucine-arginine-glutamate)-dependent mechanism for adhesion of neurons to s-laminin. J. Neurosci. *11*, 3960–3971.

Igarashi, M., Strittmatter, S. M., Vartanian, T., and Fishman, M. C. (1993). Mediation by G proteins of signals that cause collapse of growth cones. Science *259*, 77–79.

Jones, F. S., Burgoon, M. P., Hoffman, S., Crossin, K. L., Cunningham, B. A., and Edelman, G. M. (1988). A cDNA clone for cytotactin contains sequences similar to epidermal growth factor like repeats and segments of fibronectin and fibrinogen. Proc. Natl. Acad. Sci. USA *85*, 2186–2190.

Kapfhammer, J. P., and Raper, J. A. (1987). Interactions between growth cones and neurites growing from different neural tissues in culture. J. Neurosci. *7*, 1595–1600.

Lagenaur, C., and Lemmon, V. (1987). An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci. USA *84*, 7753–7757.

Lemmon, V., Burden, S. M., Payne, H. R., Elmslie, G. W., and Hlavin, M. L. (1992). Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. J. Neurosci. *12*, 818–826.

Letourneau, P. C. (1975). Cell-to-substratum adhesion and guidance of axonal elongation. Dev. Biol. *44*, 92–101.

Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217–227.

Lupa, M. T., Gordon, H., and Hall, Z. W. (1990). A specific effect of muscle cells on the distribution of presynaptic proteins in neurites and its absence in a C2 muscle cell variant. Dev. Biol. *142*, 31–34.

Miner, J. H., and Sanes, J. R. (1994). Collagen IV α3, α4 and α5 chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches. J. Cell Biol. *127*, 879–891.

Muir, D., Engvall, E., Varon, S., and Manthorpe, M. (1989). Schwannoma cell-derived inhibitor of the neurite-promoting activity of laminin. J. Cell Biol. *109*, 2353–2362.

Nies, D. E., Hemesath, T. J., Kim, J. H., Gulcher, J. R., and Stefansson, K. (1991). The complete cDNA sequence of human hexabrachion (tenascin) multidomain protein containing unique epidermal growth factor repeats. J. Biol. Chem. *266*, 2818–2823.

Nishi, T., Weinstein, J., Gillespie, W. M., and Paulson, J. C. (1991). Complete primary structure of porcine tenascin: detection of tenascin transcripts in adult submaxillary glands. Eur. J. Biochem. *202*, 643–648.

Oakley, R. A., and Tosney, K. W. (1993). Contact-mediated mechanisms of motor axon segmentation. J. Neurosci. *13*, 3773–3792.

Ochi, M., Kwong, W. H., Kimori, K., Chow, S. P., and Ikuta, Y. (1992). Reinnervation of denervated skeletal muscles by grafted dorsal root ganglion. Exp. Neurol. *118*, 291–301.

Pesheva, P., Spiess, E., and Schachner, M. (1989). J1-160 and J1-180 are oligodendrocyte-secreted non-permissive substrates for cell adhesion. J. Cell Biol. *109*, 1765–1778.

Riggs, P. (1990). Expression and purification of maltose-binding protein fusions. Curr. Prot. Mol. Biol. *10*, 16.6.1–16.6.12.

Role, L. W., Matossian, V. R., O'Brien, R. J., and Fischbach, G. D. (1985). On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes. J. Neurosci. *5*, 2197–2204.

Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M., and Scheller, R. H. (1991). Structure and expression of a rat agrin. Neuron *6*, 811–823.

Rupp, F., Ozcelik, T., Linial, M., Peterson, K., Francke, U., and Scheller, R. (1992). Structure and chromosomal localization of the mammaliam agrin gene. J. Neurosci. *12*, 3535–3544.

Sanes, J. R., Marshall, L. M., and McMahan, U. J. (1978). Reinnervation of muscle fiber basal lamina after removal of myofibers: differentiation of regenerating axons at original synaptic sites. J. Cell Biol. *78*, 176–198.

Sanes, J. R., Engvall, E., Butkowski, R., and Hunter, D. D. (1990). Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J. Cell Biol., *111*, 1685–1689.

Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R., and Yamada, Y. (1988). Laminin, a multidomain protein: the A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. J. Biol. Chem. *263*, 16536–16544.

Schnaar, R. L., and Schaffner, A. E. (1981). Separation of cell types from embryonic chicken and rat spinal cord: characterization of motoneuron-enriched fractions. J. Neurosci. *1*, 204–214.

Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedmann, T., and Taylor, P. (1986). Primary structure of *Torpedo californica* acetylcholinesterase from its cDNA sequence. Nature *319*, 407–409.

Schwab, M. E., Kapfhammer, J. P., and Bandtlow, C. E. (1993). Inhibitors of neurite growth. Annu. Rev. Neurosci. *16*, 565–595.

Smith, M. A., Magill-Solc, C., Rupp, F., Yao, Y.-M. M., Schilling, J. W., Snow, P., and McMahan, U. J. (1992). Isolation and characterization of a cDNA that encodes an agrin homolog in the marine ray. Mol. Cell. Neurosci. *3*, 406–417.

Snow, D. M., Watanabe, M., Letourneau, P. C., and Silver, J. (1991). A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Development *113*, 1473–1485.

Soreq, H., Ben-Aziz, R., Prody, C. A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Ladipot-Lifson, Y., and Zakut, H. (1990). Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C rich attenuating structure. Proc. Natl. Acad. Sci. USA *87*, 9688–9692.

Taylor, J., Pesheva, P., and Schachner, M. (1993). Influence of janusin and tenascin on growth cone behavior in vitro. J. Neurosci. Res. *35*, 347–362.

Tsim, K. W., Ruegg, M. A., Escher, G., Kroger, S., and McMahan, U. J. (1992). cDNA that encodes active agrin. Neuron *8*, 677–689.

Tuttle, J. B. (1985). Regulation of cholinergic development by target contact. Fed. Proc. *44*, 2760–2766.

Walter, J., Kern-Veits, B., Huf, J., Stolze, B., and Bonhoeffer, F. (1987). Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. Development *101*, 685–696.

Weller, A., Beck, S., and Ekblom, P. (1991). Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. J. Cell Biol. *112* 355–362.

Note Added in Proof

The data referred to throughout as P. G. Noakes et al., unpublished data, are now in press: Noakes, P. G., Gautam, M., Mudd, J., Sanes, J. R., and Merlie, J. P. (1995). Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin β2. Nature, in press.